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ELECTROANTENNOGRAM RESPONSES OF MEDITERRANEAN FRUIT FLY, *Ceratitis capitata* (DIPTERA: TEPHRITIDAE) TO TRIMEDLURE AND ITS *trans* ISOMERS

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Abstract—Electroantennograms (EAGs) of unmated laboratory-reared male and female *Ceratitis capitata* (Wiedemann) were recorded in response to the attractant trimedlure [*tert*-butyl 4(and 5)-chloro-*trans*-2-methylcyclohexane-1-carboxylate] and its four *trans* isomers. For both sexes, the magnitude of the EAG response was relatively low as compared to other previously tested compounds (i.e., plant volatiles). Dosage–response curves generated for all TML isomers revealed that flies responded to increasing dosages over a relatively narrow range (two to three log steps). Responses for both sexes peaked at ca. 10 μg dose for all isomers. Antennal response in males was greatest to the C isomer followed by the B_1, A, and B_2 isomers, while responses of females were greatest for the A isomer followed by B_1, C, and B_2. Both sexes exhibited a long recovery period for the response potential to return to baseline at doses above 1 μg for all of the isomers tested, except for B_2. The low EAG sensitivity to trimedlure and the apparent EAG selectivity to the C isomer in males are discussed in relation to the known field attractancy of males to the C, A, B_1, and B_2 isomers.

Key Words—*Ceratitis capitata*, medfly, Diptera, Tephritidae, trimedlure, electroantennogram, dosage-response.

INTRODUCTION

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), is a serious economic pest of fruit and vegetable crops throughout the world. During the past 75 years, considerable effort has been put forth to devise and improve the trapping technologies for this and other tephritid fruit fly species. Of the many compounds that have been tested as attractants for *C. capitata* (Beroza et al., 1961; Keiser et al., 1975), trimedlure (TML) [*tert*-butyl 4(and 5)-chloro-*trans*-2-methylcyclohexane-1-carboxylate] is one of the best to date and is currently used as the de facto standard for survey and detection of adult male *C. capitata*. Unlike the many pheromones or host-plant odors reported as attractants in various insects, the biological rationale as to why trimedlure, a synthetic chlorinated molecule, is attractive to male flies has remained an enigma since the serendipitous discovery of its predecessor, siglure (1-methylpropyl *trans*-6-methyl-3-cyclohexene-1-carboxylate) (Gertler et al., 1958). Trimedlure attracts both mated and unmated male *C. capitata* and has also been reported to attract virgin females in the absence of males (Nakagawa et al., 1981).

Commercial TML is composed primarily of four *trans* isomers (McGovern and Beroza, 1966) and lesser quantities of the four *cis* isomers (Leonhardt et al., 1982). The four *trans* isomers, when tested singly, elicit varying degrees of attractancy ($C \gg A > B_1 \gg B_2$), which suggests that the stereochemistry of the molecule may be an important factor (McGovern et al., 1987). Recent studies have shown the *cis* configurations of TML to be less attractive than the *trans* counterparts (McGovern et al., 1986). Although much work has been done on the isomeric chemistry of TML (McGovern and Beroza, 1966; Leonhardt et al., 1982; McGovern et al., 1986) and its attractiveness in laboratory and field tests (Beroza et al., 1961; McGovern et al., 1966, 1987), little is known about antennal sensitivity or selectivity to TML or why it elicits such a strong behavioral response, primarily in males.

Electroantennogram (EAG) recordings have been widely used to investigate detection of chemical compounds by insect antennae, but have only recently been used to study olfactory reception in tephritid fruit flies (Fein et al., 1982; Guerin et al., 1983; Van Der Pers et al., 1984; Robacker et al., 1986; Light and Jang, 1987; Light et al., 1988). These previous investigations have studied the reception of either host-plant compounds (Fein et al., 1982; Guerin et al., 1983; Light and Jang, 1987; Light et al., 1988) or identified, or putative pheromone components (Van Der Pers et al., 1984; Robacker et al., 1986, Jang et al., 1989).

The purpose of this study was to investigate the selectivity and sensitivity of adult *C. capitata* to TML and its *trans* isomers. Of special interest in this study were the questions of whether or not females possessed antennal receptors

capable of detecting these compounds and whether antennae are selectively responsive to particular isomer configurations of the molecule.

METHODS AND MATERIALS

Insects. Pupae of *C. capitata* were obtained from a laboratory mass-rearing colony maintained at the USDA, Tropical Fruit and Vegetable Research Laboratory, Honolulu, Hawaii. Upon arrival, the pupae were segregated by sex, placed in separate cages, and maintained ad libitum on sugar and water. Adult flies were tested on the second to fifth day postemergence.

Olfactory Stimuli. Commercial TML (UOP 3702) was purchased from UOP Chemical Co., East Rutherford, New Jersey, and contained the following proportions of both *trans* and *cis* isomers; *trans*: 26.9% A, 7.1% B_1, 19.7% B_2, 42.1% C; *cis*: 1.2% V, 1.3% X, 0.3% Y and W. The *trans* isomers of TML, designated as A, B_1, B_2, and C (Figure 1) according to their chromatographic retention times (McGovern and Beroza, 1966), were purified according to McGovern et al. (1987) and found to be ca. 99%+ pure by capillary GC analysis (Leonhardt et al., 1982; McGovern et al., 1986). Compounds were dissolved in spectrometric grade hexane (that was additionally distilled to 100% purity and treated with Ionox, a nonvolatile antioxidant). Serial dilutions of 100 μg/μl stock solutions of the TML blend, A isomer, and B_1 isomer were made to obtain a series of logarithmic concentrations from 1×10^2 to 1×10^{-4} μg/μl. Compounds were delivered by pipetting 1-μl aliquots onto 1 × 2-cm pieces

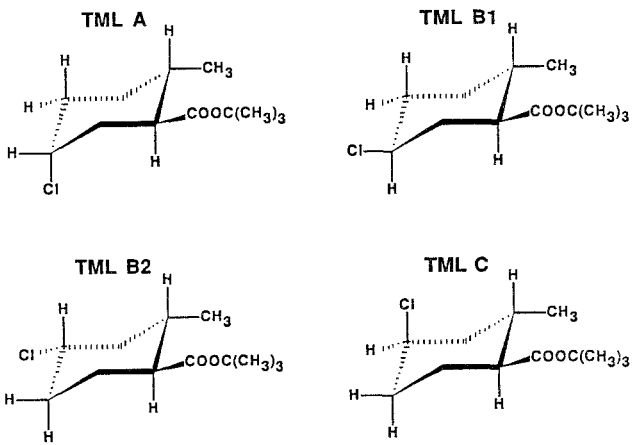

FIG. 1. Structure of *trans* trimedlure isomers.

of glass-fiber filter paper inserted into individual Pasteur pipets, which served as cartridges for the odor delivery system. Solvent was allowed to evaporate off for ca. 30 sec prior to delivery of compound over the antennae. Due to the differences in volatility of the different *trans* isomers, the less volatile isomers, B_2 and C, were tested by applying the compounds over a greater surface area of the filter paper. Based on the work by McGovern et al. (1966) on the relative volatility of these isomers, the B_2 and C isomers evaporate ca. 6.6× and 3.5× slower than the A isomer. To compensate for this difference, these compounds were diluted to obtain stock concentrations of 15.2 μg/μl and 28.6 μg/μl, respectively, and serially diluted over seven log steps. The B_2 and C isomers were applied to the filter paper at the same microgram dose as the A and B_1 isomers (i.e., 100 μg to 1×10^{-4} μg), but spread over a proportionally greater surface area (i.e., ca. 6.6× and 3.5× the size of the A isomer). This procedure "equalized" the effective volatile dose of each isomer, which was subsequently presented to the antennae. Either 6.6 μl (B_2) or 3.5 μl (C) was aliquoted onto the filter paper to achieve this effect. The diluting solvent was allowed to evaporate for 2 or 3 min before testing as determined with hexane controls using the larger volumes. These controls were found to give no higher response than the previous 1-μl volume controls.

Electrophysiological Recording Technique. Electroantennogram (EAG) techniques used in this study were identical to previously published techniques utilizing glass capillary Ag–AgCl electrodes (Light and Jang, 1987). Intact flies were immobilized by a yoke in a Plexiglas block. The recording electrode was inserted into the apical region of the terminal antennal segment, the funiculus; the indifferent electrode was positioned into the hemocoel of the cranial cavity. The signal was amplified 100× by a Grass P-16 microelectrode preamplifier and viewed on a Nicolet model 4094 digital storage oscilloscope. EAG deflections were measured from the calibrated screen and stored on floppy disks.

Odor Delivery System. The odor delivery system and stimulation apparatus were essentially the same as that described by Light (1983). A constant flow (1.0 liter/min) of charcoal-filtered and humidified compressed air was passed over the antenna through a disposable plastic pipet tip positioned ca. 1 cm from the antenna. A three-way solenoid valve diverted the purified air to the stimulus cartridge where the test odor was purged from the pipet tip and over the antenna. Stimulation was for 1 sec. For each compound, the order of presentation was always from lowest to highest dosage. A minimum of 3–4 min of clean air preceded and followed each stimulation. This time period allowed for full recovery of the antenna at the highest (100 μg) dose tested.

Experimental Procedure. EAGs were recorded from at least five individual flies of each sex for the TML commercial blend and for each of the isomers over a seven log dose range. "Control" stimulations containing either 1, 3.5,

or 6.6 μl of the hexane solvent and "standard" stimulations containing fresh cartridges of 1 μl of 1% hexan-1-ol (100% purity, Aldrich Chemical Co.) dissolved in hexane were presented between each appropriate series of compounds. EAGs were normalized to the standard stimulation by measuring the maximal amplitude of the negative deflection (−mV) during the stimulation period elicited by a given stimulus, subtracting the amplitude of the response to the preceding hexane control, and then dividing by the mV response to the accompanying 1% hexan-1-ol standard (from which the hexane control was also subtracted) to obtain the percent of the standard response (the standard being a 100% response in each case). This "normalization" minimized the observed variability in: (1) absolute responsiveness among preparations, (2) the time-dependent variability in antennal responsiveness, and (3) allowed for relative comparison of responses between sexes (Payne, 1975; Light, 1983; Dickens, 1984). Hexan-1-ol was chosen as a standard based on its relatively constant response as measured on many individuals in previous studies (Light and Jang, 1987; Light et al., 1988; Jang et al., 1989). The mean mV responses to standard stimulations measured for each sex in this study were 1.09 ± 0.08 mV for males and 1.14 ± 0.11 mV for females. Mean normalized TML responses were statistically compared using *t* tests and the nonparametric Mann-Whitney U test (Snedecor and Cochran, 1967). A "threshold" EAG response (or the minimal effective dosage needed to produce a reliably detectable response) was arbitrarily defined as the stimulus dosage at which the lower limit of the standard error to the test stimulus did not overlap with the upper limit of the standard error for the response to control (Light, 1983; Dickens, 1984). The upper limit of the standard error to the hexane control was calculated as 9.3% of standard and 5.5% of standard, respectively, for males and females.

The period of time necessary for the EAG depolarization to return to the baseline trace level for a given stimulus was defined as the recovery period. To evaluate the effects of the stimuli on antennal recovery period, the percent recovery was calculated by measuring the positive mV recovery 3 sec after the end of the stimulus, and dividing it by the maximum depolarization (see Figure 3). Comparisons of the mean percent EAG recovery at three seconds post-stimulus were made using ANOVA and Duncan's multiple-range test (Duncan, 1955).

RESULTS

Sensitivity. Dosage–response curves of male and female *C. capitata* to the commercial mixture of TML and the *trans* isomers are shown in Figure 2. In general, flies responded to increased dosages of the commercial blend and isomers A, B_1, and C over a relatively narrow dosage range (two to three log

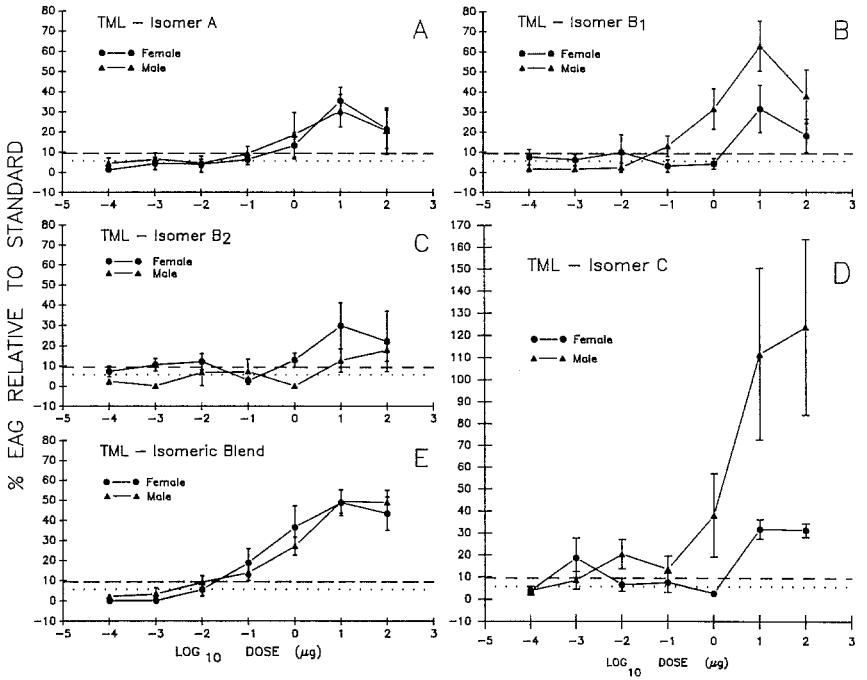

FIG. 2. Dosage–response curves of male (triangle) and female (circle) *C. capitata* to the commercial blend and individual *trans* isomers of trimedlure. Points represent the mean response ± SE of at least five male and female flies relative to a standard (1 μl dose of 1% hexan-1-ol), and the horizontal lines (dashes, males; dots, females) delineate threshold levels (one SE of control above zero, see Methods and Materials) above which responses are significantly greater than control ($P < 0.05$). (A) Isomer A; (B) isomer B_1; (C) isomer B_2; (D) isomer C; (E) commercial blend.

steps). Responses peaked at ca. 10 μg for each of these isomers and for both sexes.

The antennae of both sexes showed similar EAG responses to the commercial blend (Figure 2E), with no significant differences between the sexes observed at any of the doses tested. Threshold responses appeared to occur at less than the 0.1-μg dose for both sexes. Increased responses were evident over the dose span from above 0.01 μg to 10 μg, then plateaued at 100 μg.

Isomer A of TML elicited similar EAG response curves for males and females (Figure 2A, Table 1). The threshold response occurred between 1.0 and 10 μg for males and females. Antennae of both sexes showed a maximum response to the 10-μg dose.

Dose–response curves to the B_1 isomer differed between the sexes in

TABLE 1. EAG THRESHOLD AND RESPONSE MAXIMA FOR MALE AND FEMALE *Ceratitis capitata*

	EAG threshold (μg)		Maximum EAG response ± SE	
	Male	Female	Male	Female
Blend[a] (99+%)	<0.1	<0.1	49.2 ± 15.9	48.7 ± 6.5
Isomer[b] A (99+%)	>1.0	>1.0	30.5 ± 8.2	35.6 ± 6.8
Isomer B_1 (97%)	>0.1	>1.0	62.9 ± 12.6	31.6 ± 12.2
Isomer B_2 (99+%)	>10.0	>10.0	17.9 ± 5.6	29.8 ± 11.4
Isomer C (99+%)	>0.1	>1.0	123.8 ± 39.9	31.7 ± 4.6

[a] Composition of commercial trimedlure (UOP 3702) *trans*: 26.9% A, 7.1% B_1, 19.7% B_2, 42, 1% C; *cis*; 1.2% V, 1.3% X, 0.3% Y and W.
[b] Preparation of isomers as in McGovern et al. (1987).

threshold level, slope, and dynamic response range (Figure 2B, Table 1). Males had a lower threshold (>0.1 μg vs. >1 μg) and responses increased over a broader stimulus range than the female curve. Isomer B_1 elicited significantly greater ($P < 0.05$) responses to both the 1-μg and 10-μg doses from male than female antennae.

Neither sex responded significantly above threshold to the B_2 isomer over the majority of the dose range tested (Figure 2C). Only the females at the 10-μg dose and males at the 100-μg dose showed responses above threshold. However, relative responses were still quite low compared to the other isomers, even with compensations made for the low volatility of this isomer.

EAG responses of male *C. capitata* to the C isomer were significantly greater than those of females and were also the highest recorded among all the compounds tested (Figure 2D, Table 1). Male antennae responded above threshold at the >0.1-μg dose, while female antennae were a log step less sensitive. Maximum response was seen at the 100-μg dose for both sexes.

Selectivity. Comparing the responsiveness of both sexes to the four isomers, antennal response in males was greatest to the C isomer followed by the B_1, A, and B_2 isomers. Females exhibited maximum responses to the A and B_1 isomers followed by the C and B_2 isomers (Table 1). Differences between the sexes were most evident for the C and B_1 isomers, in both cases males having a greater response over a two to three log dose range than females (Figures 2D and B).

Recovery Period. The antennae tested exhibited a long recovery period to baseline after presentation of TML or its isomers at concentrations above 1 μg (Figure 3 and Table 2). The antennal recovery period at these higher doses was prolonged by all isomers except the B_2 isomer. Recovery periods considered

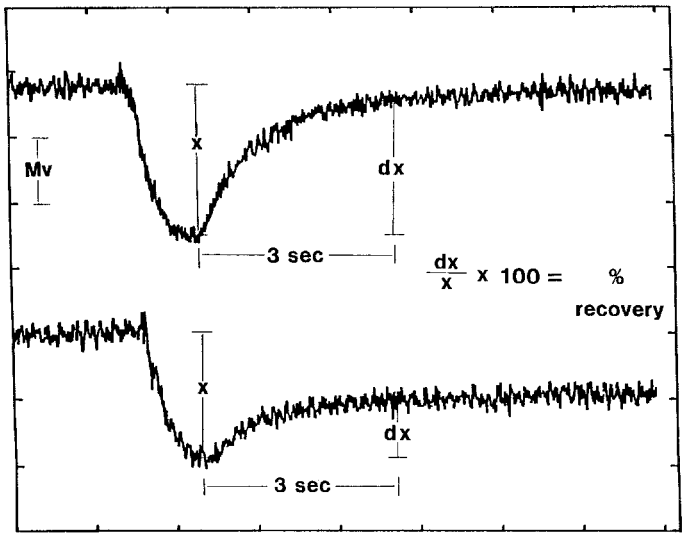

FIG. 3. Electroantennogram recording showing "normal" (hexan-1-ol standard) and "long" (isomer C, 10-μg dose) recovery of antennal receptors. Recovery was analyzed by calculating the percent change between the maximum deflection (X) and the recovery of the stimulation trace towards baseline (dX) at 3 sec after stimulus.

"normal" were those in which the trace of the oscilloscope recording returned to the approximate baseline within ca. 3–4 sec of the end of the stimulus delivery. Stimulation traces exhibiting "long recovery" did not return to baseline within the field of view of the oscilloscope screen (ca. 10 sec). Most antennae exhibited "long recovery" at a concentration of 10 μg, although a few were affected at lower doses. Analysis of the relative recovery rates for males after three seconds at the two highest doses tested showed significantly more long recovery for the blend, A, B_1, and C isomers than for the B_2 isomer (Table 2). Females responded similarly except for the C isomer, which was significant only at the highest dosage tested. EAGs showing long recovery did, however, return to baseline well within the minimum time period chosen between presentations of the compounds (3–4 min). The B_2 isomer and the hexan-1-ol standard exhibited "normal" recovery in all cases.

DISCUSSION

Both males and females exhibited EAG responses above threshold to the TML commercial blend and three (A, B_1, and C) of the four *trans* isomers of TML, indicating that *C. capitata* has receptor populations capable of detecting

TABLE 2. MEAN PERCENT RECOVERY TO BASELINE OF *C. capitata* ANTENNAL RESPONSES TO TRIMEDLURE AND ITS *trans* ISOMERS 3 SEC AFTER STIMULATION

Compound (μg)	Mean percent recovery after 3 sec relative to maximum (−mV) deflection	
	Male	Female
TML A		
0.1	94.1 ± 4.3 abc[a]	105.2 ± 3.1 a
1	79.5 ± 3.2 cd	87.2 ± 5.8 abc
10	47.3 ± 10.2 f	45.7 ± 9.1 efg
100	55.4 ± 7.5 ef	44.5 ± 9.4 efg
TML B_1		
0.1	100.4 ± 8.4 ab	103.1 ± 7.6 ab
1	93.8 ± 9.5 abc	77.7 ± 6.3 abcd
10	58.8 ± 7.7 ef	43.0 ± 7.9 fg
100	56.2 ± 2.4 ef	30.2 ± 6.5 g
TML B_2		
0.1	72.1 ± 16.7 bcd	100.0 ± 0.0 ab
1	96.2 ± 3.8 abc	95.5 ± 3.4 abc
10	76.1 ± 8.0 cd	85.5 ± 6.5 abcd
100	95.6 ± 3.2 abc	97.1 ± 3.2 abc
TML C		
0.1	80.3 ± 8.8 cd	89.3 ± 10.7 abc
1	80.7 ± 5.4 cd	91.4 ± 8.6 abc
10	58.3 ± 5.0 ef	76.8 ± 8.6 abcd
100	51.7 ± 7.3 ef	53.7 ± 6.6 efg
TML Blend		
0.1	87.9 ± 8.9 abc	89.9 ± 7.2 abc
1	88.2 ± 5.3 abc	90.9 ± 14.6 abc
10	52.3 ± 5.0 ef	67.0 ± 16.9 cdef
100	56.0 ± 4.2 ef	56.4 ± 12.9 defg

[a] Values followed by the same letter are not significantly different from each other using Duncan's multiple-range test ($P < 0.05$). Means were calculated from at least five individuals of each sex.

these compounds. However, the relative magnitudes of these EAG responses to these compounds were relatively low compared to the hexan-1-ol standard and most plant volatiles previously tested, which elicit EAGs ranging from ca. 100% to 300% of standard at a 100-μg dose (Light et al., 1988). These plant volatiles include various monoterpenes, aliphatic esters, primary alcohols, and aldehydes; in particular, the general "green-leaf" volatiles, appear to be more potent EAG stimuli to *C. capitata* antennae. The exception was the male antennal response to the C isomer at the two highest doses, which elicited responses comparable to many of the plant volatile compounds and which elicited a significantly higher response than either the females to the same compound or

either sex's response to the A, B_1, or B_2 isomers. Significantly greater ($P < 0.05$) EAG responses between sexes were also evident in males for the B_1 isomer (at doses of 1.0 and 10 μg).

The magnitude of the EAG response is thought to be representative of the overall population size of the antennal receptors responding to a particular olfactory stimulus (Kaissling, 1971; Payne, 1975; Nagai, 1981, 1983; Mankin and Mayer, 1983; Mayer et al., 1987). Preliminary single-cell recordings (Dickens, Light, and Jang, unpublished observations) indicate the presence of cells responsive to the TML blend that are capable of detecting differences in relative doses. These TML-responsive receptor neurons were recorded from seven of the 23 cells in our preliminary sampling of sensilla distributed over various regions of the funiculus (Dickens et al., 1988). The low numbers of TML-responsive antennal receptor neurons compared to those responding to plant odors encountered in these preliminary single-cell recordings correlate with the relatively low EAG responses to most isomers, suggesting that there are low densities or small overall populations of TML receptors present. Therefore, we conclude that *C. capitata* antennae may possess small populations of receptors for the A, B_1, and B_2 isomers compared to those responsive to other semiochemicals such as selected plant volatiles. Males do, however, possess larger populations of antennal receptors responsive to the C isomer than for the other three *trans* isomers, while females may have similar numbers of receptors for the A, B_1, and C isomers. This difference suggests that males may possess specific receptors that are responding to the C isomer but are not part of the population that responds to the other isomers and may, in part, explain why the C isomer is the most active of the individual isomers in field tests. Males show a striking behavioral attraction to TML, whereas neither sex is attracted to most individual plant odors or green-leaf odors (Beroza and Green, 1963; Cunningham, unpublished observations). Primarily virgin females rather than mated females have been reported to be attracted to TML in the field (Nakagawa et al., 1981). Yet in preliminary EAG recordings to mated females (Jang, unpublished observations) we find little difference in EAG response to trimedlure or its *trans* isomers over that reported in this study to virgin females. This may indicate that other physiological states may influence perception and/or elicitation of a behavioral response to certain semiochemicals (Davis, 1984; Blaney et al., 1986).

The chemoreceptive basis for TML being a potent nonpheromonal attractant for *C. capitata* is due not only to the presence of populations of antennal receptors for these isomers, but also to the specificity and sensitivity of receptors and ultimately the CNS to specific configurations of the TML molecule. McGovern et al. (1987) suggested that the stereochemical configuration of the TML molecule was an important factor in its attractiveness based on extensive

field testing of the four *trans* isomers of TML, which showed the C isomer to be most attractive, followed by the A, B_1, and B_2 isomers, respectively. For maximal attraction in the field, they considered the axial positioning (isomers C and A) of the 4- or 5-position chlorine atom of TML to be more suitable than an equatorial conformation (isomers B_1 and B_2), and that the diequatorial position of the 1 and 2 substituents on the cyclohexane ring (*trans*) is preferable to an axial-equatorial position (*cis*). Electrophysiological evidence from this study suggests that the C and B_1 isomers are more potent antennal stimulants for males than the A or B_2 isomers at the same dose, while very little difference in EAG response to the TML isomers by the females was observed.

Although the precise significance is not known, the "long recovery" of the medfly antennal receptor neurons to high doses of TML suggests a strong interaction of the molecules with their receptors, which may be indicative of the relative "affinities" or "stickiness" of the compounds (Roelofs et al., 1969; personal communication), or slow "inactivation" of the receptor complex and/or the TML molecule (Kaissling, 1969, 1974). We found that isomers of TML that have been reported to be the most attractive (C, A, B_1) exhibited longer recovery than that of low attractancy (B_2) isomer or plant volatiles (Light et al., 1988). This difference in recovery as seen between behaviorally "active" compounds such as trimedlure and pheromones, and other more "general" odors may have a functional significance in how insects interpret chemical cues. An in-depth discussion of chemoreceptive recovery and inactivation is presented by others elsewhere (Kaissling, 1971, 1986).

We conclude from these studies that (1) both male and female *C. capitata* do possess populations of antennal receptors capable of detecting trimedlure, (2) there are differential population levels of antennal receptors responsive to different configurations of the TML molecule, and (3) based on these results, it appears that there is a relationship between differential population levels of receptors and previously published field attractancy to these same compounds. Single sensillum recordings have been initiated that may provide additional information useful in determining the precise structure–activity relationships of the TML geometric and optical isomers with antennal receptors and, perhaps, insights as to why particular isomers and/or enantiomers are more attractive than others. Future single-cell recordings on the sensitivity, specificity, and long recovery phenomena of receptor neurons from male and female *C. capitata* may reveal the mechanisms by which these isomers are detected. These studies may also reveal the neural basis of the observed behavioral differences between the sexes for both TML and host plant odors.

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